APPLICATION OF CELLULAR MICROENCAPSULATION TO WASTEWATER NITROGEN REMOVAL

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Bacterial Microencapsulation for use in Wastewater Nitrogen Removal. (May 2013)

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Microencapsulation of cells is a technique in which cells are enclosed in microscopic spheroids of a desired substance, with applications in many fields, such as biomedical engineering. In this study, a microfluidic approach to encapsulate bacteria (nontoxic to humans) in polyethylene glycol-diacrylate (PEG-DA) spheroids is investigated, particularly its potential application in a multistage bacterial process to remove Nitrogen from wastewater. A mixture of the bacteria and PEG-DA solution is pumped through channels in a microfluidic device. Mineral oil is also pumped simultaneously into microfluidic channels. PEG-DA microdroplets will be generated due to the shear focusing effect of the mineral oil on the PEG-DA mixture at the cross junction in a microfluidic device, and cross-linked immediately in a straight channel by a time of UV exposure around 0.1 milliseconds. The cross-linked PEG-DA spheroids will be extracted from the mineral oil phase by centrifugation. This approach is expected to produce PEG-DA spheroids with diameter of 50 μm to 300 μm. In the case of the multi-step bacterial process, a procedure for multiple-encapsulation (yet another encapsulation of already cross-linked PEG-DA microspheres) will also be examined. The number and viability of cells in the PEG-DA spheroids is subsequently investigated.

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NOMENCLATURE

AOB	Ammonia Oxidizing Bacteria
Annamox	Anaerobic Ammonia Oxidizing Bacteria
Micro-channel	Tubular structure with a diameter on the micro-scale through which liquid can be pumped for use in microfluidic devices
Microencapsulation	Well-known method in biomedical science in which cells are enclosed in microscopic spheroids of a desired substance
PEG-DA	Poly (ethylene glycol) diacrylate
PDMS	Polydimethylsiloxane
UV	Radiation in the ultraviolet spectrum (wavelength of 10 nm to 400 nm)

CHAPTER I

INTRODUCTION

Cell-containing microspheres are widely used as building blocks in many biomedical applications such as tissue engineering, cell-based biosensors, and encapsulated cell delivery. These applications require uniform microsphere dimensions and morphology with high cell viability. Live cells are typically encapsulated in biodegradable polymers such as alginate, polyethylene glycol (PEG), and collagen that are highly porous, allowing adequate transport of nutrients and oxygen to the cells. **[1]** This study deals with the encapsulation of bacterial cells in PEG-DA (diacrylate) microspheres.

One potential application of bacterial micro-encapsulation in PEG-DA is an implementation in the bacterial removal of nitrogen pollutants from wastewater. Wastewater nitrogen pollution is a pressing issue which presents environmental threats including toxicity to aquatic biota and the overstimulation of phytoplankton growth, and human health implications including the blue-baby syndrome and the formation of carcinogenic compounds. [2] The proposed method is a process involving two distinct classes of bacteria named Ammonia Oxidizing Bacteria (AOB). The first bacteria to act in the two-stage chemical process requires oxygen to survive, while the second, consisting of Anaerobic Ammonia Oxidizing bacteria (Annamox), favors oxygen-deprivation. There is a clear mismatch in optimal conditions for each of the two bacteria. Thus the advantage of microsphere encapsulation here would be to create a precisely controlled environment for the bacteria to thrive separately in their desired conditions by creating separate areas of high cell concentration for each type of organism. This is achieved by

microencapsulation of the cells in a material called PEG-DA (Poly (ethylene glycol) diacrylate). The bacteria are to be localized and encapsulated in distinct layers in a single microsphere, with the outer layer containing the aerobic bacteria, and the inner the Annamox culture, with the hopes that this would create a better environment for both bacteria with higher efficiency in Ammonia reduction.

The objectives of this study are then two-fold: the first is to develop the fabrication processes for microsphere encapsulation of bacteria and the second is to attempt the double-encapsulation procedure with the goals of retaining high cell viability and creating optimal conditions for the bacteria to thrive.

CHAPTER II

METHODOLOGY

Overview

This proposed work requires two encapsulation layers, with one of the two types of bacterial culture constrained to each. The best approach to this is to first create PEG-DA microspheres containing the Annamox bacteria. These are subsequently inserted into a liquid PEG-DA mixture containing the second bacterial culture. Another microencapsulation procedure is then completed on this mixture, yielding the final product.

In the development of the bacterial double-encapsulation procedure, two approaches are examined. The first utilizes microfluidic devices to generate PEG-DA droplets containing bacteria to be encapsulated which are then cross-linked under UV radiation. The other method lithographically defines PEG-DA micro-squares to form the interior encapsulation layer. The outside layer is achieved through the use of PDMS microtemplates.

The microfluidic approach: creating a microfluidic device

Microscopic PEG-DA droplets can be generated to encapsulate the bacteria through the use of a microfluidic device, or a device which can control the flow of liquid on the micro-liter scale through micro-channels. The process for creating and using such devices has been thoroughly developed and refined.

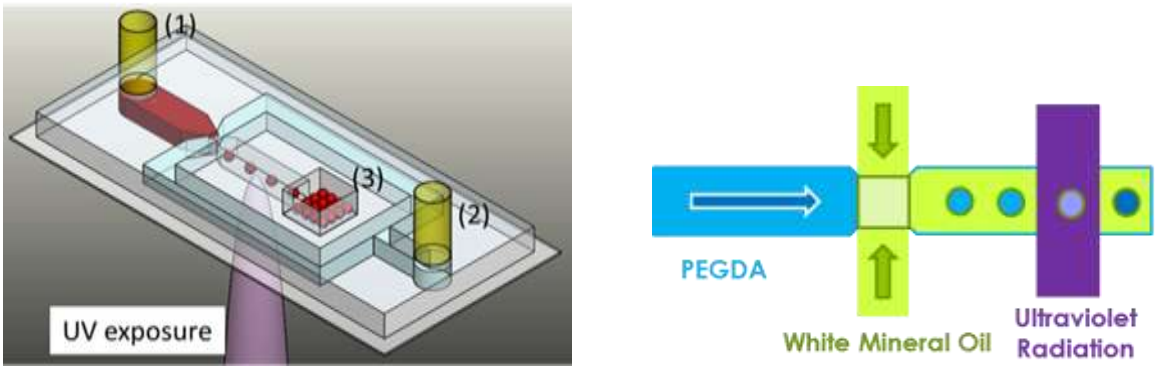
For this application, the devices are crafted out of PDMS (Polydimethylsiloxane). First, a mold is created on a Silicon wafer which PDMS will solidify around to create channels. In order to do

this, a schematic of the micro-channel layout is created on CAD or some other drawing program then printed as a mask through <www.pageworks.com>, which allows resolutions of 10-15 μm. A silicon wafer coated with SU8-2075 negative photoresist to the desired channel height is exposed and developed according to manufacturer instructions.

After the creation of the mold, a mixture in a 9:1 ratio of liquid PDMS and binding agent are poured around the mold (air bubbles are removed using a vacuum -pump) and baked at a temperature of 65℃. The PDMS will solidify around the mold, and grooves are created which will form the body of the micro-channel. The solid PDMS is subsequently plasma-treated in order to break the surface bonds on the material, and then placed in contact with a thin glass sheet. This allows the PDMS to bond to the surface of the glass which forms the remaining side of the micro-channels. Finally, the device is baked once again to return the interior surfaces of the micro-channels to a hydrophobic state.

The microfluidic approach: encapsulation procedure

In order to create the PEG-DA microspheres used to encapsulate bacteria, microfluidic devices created through the process described in the previous section are utilized. A mixture of the live bacterial culture (separated from the original nutrient medium by centrifugation), liquid PEG-DA, and a photo-initiator, which will cross-link (solidify) the PEG-DA when exposed to ultra-violet radiation, is created. (Prolonged light exposure must be avoided after creation of the mixture, as it could begin to crosslink before the desired time).



(a) **(b)**

Figure 1: Microfluidic encapsulation process
(a) Schematic diagram of microfluidic device for the creation of PEG-DA microspheres. Liquid PEG-DA enters through channel labeled *(1)*, while oil is pumped through *(2)*. Droplets are cross-linked in a UV- irradiated region of the channel, then collected in a reservoir, labeled *(3)*.

(b) Illustration of droplet generation in micro-channel cross-section by shear-focusing effect of oil on PEG-DA inflow. Droplets are led under UV radiation in outflow channel. Flow-rates of the PEG-DA and white mineral oil are modulated to calibrate droplet size.

As shown in Figure 1(a), a completed microfluidic device is then used to generate micro-scale droplets of the mixture which are then cross-linked by a short exposure to ultra-violet radiation while passing through the channel, and collected in a reservoir. In order to achieve this, the liquid mixture and white mineral oil mixed with 4% SPAN surfactant (in order to prevent mixing with the PEG-DA) are pumped through separate channels in the micro-fluidic device. Depicted in Figure 2(b), the channel carrying the oil intersects the PEG-DA carrying channel from two sides. When the flow rates are properly calibrated, the shear-force of the oil on the liquid stream will generate droplets of the mixture in the outflow channel, the size of which can be modulated by calibrating the flow rates of the liquids. The droplets are led across a region irradiated by UV light, initiating crosslinking in the PEG-DA. The microspheres are then led to a reservoir where they can be collected.

The lithography and microtemplate approach

The other method examined in this study encapsulates bacteria in two different manners for the inside and outside encapsulations. The inside encapsulation of the Annamox bacteria is achieved through photolithography, or by irradiating a thin layer of PEG-DA solution-cell mixture through a printed mask to generate cross-linked areas of the desired size which can then be removed and replaced into suspension with AOB for the second encapsulation.

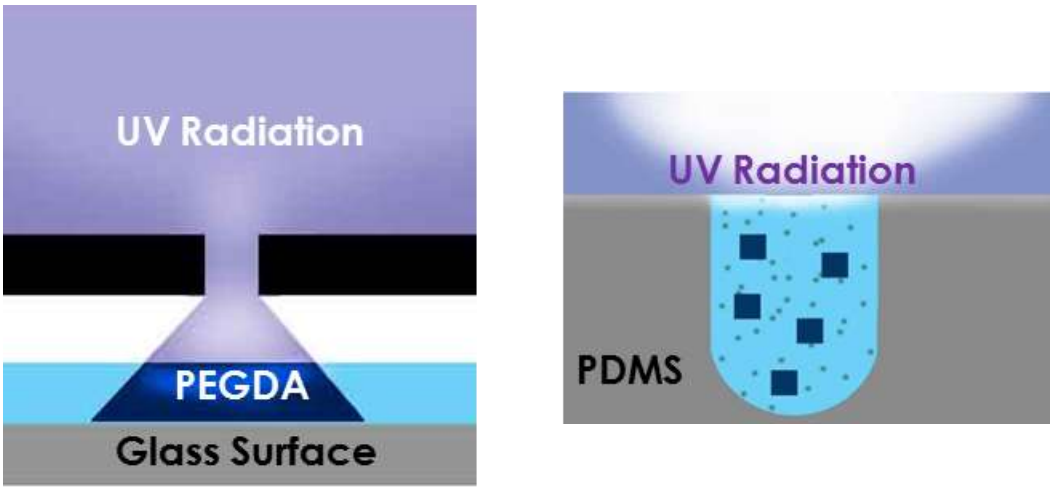


Figure 2: Lithographic and micro-template encapsulation process
(a) Illustration of lithographic micro-square generation for interior encapsulation. Localized UV irradiation on PEG-DA layer due to the mask triggers cross-linking in desired areas.

(b) Illustration of micro-template encapsulation for outer layer. PEG-DA solution is placed into bullet-shaped microfluidic mold, then exposed to UV radiation for cross-linking.

As depicted in Figure 2(a), the PEG-DA and Annamox mixture is manually spread across a thin glass sheet. In order to properly distribute the liquid, it is necessary to plasma-treat the glass to create a hydrophilic surface which is conducive to such a procedure. The mask, which features an array of square openings of width 75 μm, once again printed through

<www.pageworks.com>, is suspended close to the surface. This arrangement is then irradiated by UV, which passes through the openings of the mask to crosslink areas of the PEG-DA mixture. For this step, the expected result is marginally uniform areas of cross-linked PEG-DA, which are attached to the glass surface beneath, allowing them to be washed clean of the remaining solution and placed into suspension with the AOB bacteria.

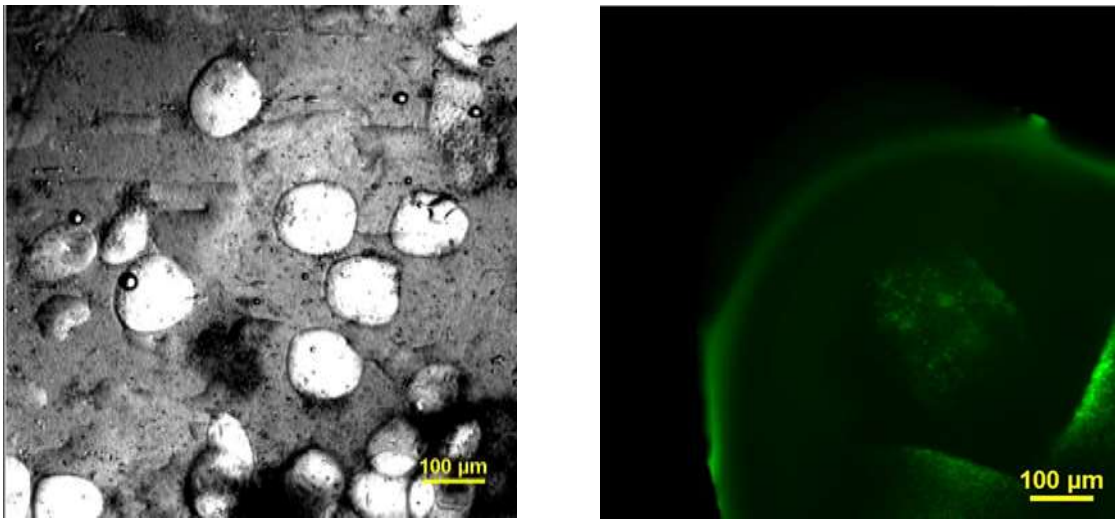
The procedure for the second layer of encapsulation, shown in Figure 2(b), utilizes an array of microfluidic molds, or micro-bullet template, to trap the liquid mixture for cross-linking. These molds are created in PDMS through the process detailed previously as creating a microfluidic device. In order to properly facilitate diffusive penetration through the material, the diameters of the cavities are maximally on the order of 800 μm. Due to the comparatively large diameter of the molds, it is possible for them to be manually loaded by once-again plasma-treating the PDMS surface and spreading the liquid mixture across the surface of the array to fill the cavities. Once this is complete, UV radiation is applied for approximately 5 to 8 seconds, resulting in full crosslinking in the loaded cavities. The cross-linked PEG-DA capsules can then be removed manually by scraping the surface of the array, and placed into fresh cell medium.

CHAPTER III

RESULTS

The microfluidic approach

Single encapsulations were performed on E. Coli bacteria, successfully utilizing the microfluidic approach with high viability. Uniformly shaped microspheres were obtained in the desired diameter range around 50 μm. Cell viability was checked post-procedure utilizing dead-alive dye with fluorescence microscopy, and it was found that cells remained viable inside the microcapsules. PEG-DA microspheres produced through oil-shearing are shown in Figure 3(a).



(a) **(b)**

Figure 3: Micro-capsules
(a) PEG-DA microspheres generated through micro-fluidic oil-shearing. Cells are encapsulated, however they are not visible without florescence microscopy due to refraction effects and insufficient magnification.

(b) PEG-DA micro-bullet encapsulation of E. Coli bacteria viewed under florescence microscopy using dead/alive dye. Viable cells are visible glowing green inside the capsule.

For the purpose of double-encapsulation, however, it was determined that the microfluidic approach using meniscal shear force to generate droplets is impractical with the current laboratory setup. One issue that arises with this approach is its low yield. Because in the application of nitrogen-removal from wastewater tanks, high amounts of microcapsules are needed for dispersal in a large tank, the slow droplet generation of this method means a clear disadvantage. Further problems arise due to the white mineral oil used as the droplet separator in this method, as it is difficult to remove cross-linked microspheres from it, and its presence in the mixture yields suboptimal conditions for cross-linking the outside encapsulation layer. Thus, while the single encapsulation using this method yielded promising results, its use in the double encapsulation procedure is not a viable option.

The lithography and microtemplate approach

Single encapsulations were completed with this method, this time utilizing the microfluidic molds. As expected, the molds produced micro-bullets with a diameter on the order of 800 μm. High viability for E. Coli bacteria was observed inside the capsules using florescence, even after incubation post-encapsulation for more than 72 hours. PEG-DA micro-bullets produced with this approach are shown in Figure 3(b).

The generation of an inside layer of encapsulation utilizing the mask approach presented problems, however. Cross-linked fragments of PEG-DA with low structural integrity and uniformity were obtained using this method. Furthermore, the expectation that the cross-linked PEG-DA pieces would remain attached to the glass surface below proved to be false, rendering it difficult to remove the remaining liquid mixture without also removing the microcapsules.

There are two main reasons for the suboptimal performance of the mask approach to the inside encapsulation. Firstly, it is difficult, given our current laboratory setup, to obtain a uniform layer of the PEG-DA mixture across the surface of the glass that is thin enough to easily allow the UV radiation to penetrate to the far side in order to attach the PEG-DA fragments to the surface. Second, the separation between the mask and the surface of the liquid layer was far too great to allow cross-linking to occur normally. Due to diffraction effects yielded by the small diameters of the openings in the mask, the UV light that passes through is of a much lower intensity than was anticipated. In the current experiments, a separation distance of approximately 1 mm was attained, however this is still far too great for scattering to permit significant cross-linking on the surface of the PEG-DA solution.

CHAPTER IV

CONCLUSIONS

Cellular microencapsulation has great potential in increasing the efficiency of bacterial wastewater Nitrogen removal processes, not only in protecting cells from toxicants, but also in providing a suitable environment for multiple cell types with contrasting needs to coexist. In order to achieve this goal, Annamox bacteria and AOB must be encapsulated in distinct layers. To achieve this goal, two different approaches were pursued.

The first method, utilizing a microfluidic droplet generation device, is widely used, and its success in the single encapsulation showed promise for this application. However, when applied to the second layer of encapsulation, the low droplet production rate coupled with the incompatibility of white mineral oil with PEG-DA crosslinking leaves the method impractical for this purpose.

The second approach utilizes contact lithography to crosslink a PEG-DA layer for the first encapsulation, and a micro bullet template for the double-encapsulation. The procedure for the interior layer encapsulation presents some challenges, however the micro-bullet template were used separately to encapsulate cells with high viability. The difficulties with the lithographic method can be overcome by optimizing the exposure dosage by increasing the UV exposure time to counteract the effect of intensity reduction due to scattering.

In conclusion, though thus far the attempts to perform a double-encapsulation have not met with complete success, the procedure's completion hinges on the rectification of a few minor technical details. Further study along this direction will then be focused on solving these problems, and performing tests to determine whether double encapsulation permits high cell viability. Once this is accomplished, the process will be applied to wastewater nitrogen removal with microencapsulated bacteria.

REFERENCES

[1] **Sungmin Hong , Hui-Ju Hsu , Roland Kaunas & Jun Kameoka**, (2012), *Collagen microsphere production on a chip*. Lab Chip, 12, 3277-3280

[2] **Ni-bin Chang (2011)**, *Making a Progress to Speed up the Nitrification and Denitrification Processes in Novel Biosorption Activated Media: Can Archaea be in Concert with Anammox?* J Bioprocess Biotechniq 1:103e doi: 10.4172/2155-9821.1000103e